Removal of antibiotic resistance genes in two tertiary level municipal wastewater treatment plants

McConnell, Mandy M.; Hansen, Lisbeth Truelstrup; Jamieson, Rob C.; Neudorf, Kara D.; Yost, Christopher K.; Tong, Anthony

Published in:
Science of the Total Environment

Link to article, DOI:
[10.1016/j.scitotenv.2018.06.212](https://doi.org/10.1016/j.scitotenv.2018.06.212)

Publication date:
2018

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):
McConnell, M. M., Hansen, L. T., Jamieson, R. C., Neudorf, K. D., Yost, C. K., & Tong, A. (2018). Removal of antibiotic resistance genes in two tertiary level municipal wastewater treatment plants. *Science of the Total Environment*, 643, 292-300. <https://doi.org/10.1016/j.scitotenv.2018.06.212>

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

1 **Removal of Antibiotic Resistance Genes in Two Tertiary**
2 **Level Municipal Wastewater Treatment Plants**

3

4

5 Mandy M. McConnell^a, Lisbeth Truelstrup Hansen^{b*1}, Rob C. Jamieson^a, Kara D.
6 Neudorf^b, Christopher K. Yost^c, Anthony Tong^d.

7

8 ^a Department of Civil and Resources Engineering, Dalhousie University, 1360 Barrington
9 Street, Halifax B3H 4R2, Canada

10 ^b Department of Process Engineering and Applied Science, Dalhousie University, 1360
11 Barrington Street, Halifax B3H 4R2, Canada

12 ^c Department of Biology, University of Regina, 3737 Wascana Parkway, Regina, SK S4S
13 0A2, Canada

14 ^d Department of Chemistry, Acadia University, 6 University Avenue, Wolfville, Nova
15 Scotia, Canada B4P 2R6

16 *Corresponding author at: National Food Institute, Technical University of Denmark,
17 Denmark.

18 E-mail address: littr@food.dtu.dk (L. Truelstrup Hansen).

19 ¹ Present address: National Food Institute, Technical University of Denmark, DK-2800
20 Kongens Lyngby, Denmark.

21

Abstract

Raw wastewater can contain high levels of antibiotic resistance genes (ARGs), making municipal wastewater treatment plants (WWTPs) critical for the control of the release of ARGs into the environment. The objective of this study was to investigate how individual treatment steps in two tertiary WWTPs affected the removal (copies/mL) and relative abundance of ARGs (copies/copies 16S rRNA genes). Nine ARG markers, representing resistance to commonly used antibiotics, as well as one integron gene (*int1*) to assess ARG mobility potential, were quantified using quantitative real-time PCR (qPCR). Both WWTPs met provincial effluent regulations for removal of carbonaceous oxygen demand (CBOD₅) and total suspended solids. Eight of the ten ARG markers (*int1*, *sul1*, *sul2*, *tet(O)*, *ermB*, *bla*_{CTX-M}, *bla*_{TEM}, *qnrS*) were detected in all samples. In contrast, *mecA* was detected intermittently and *vanA* remained below the detection limit in all samples. The total ARG marker abundances decreased by log 1.77 ($p < 0.05$) in the plant using an aerated lagoon (AL), and by 2.69 logs ($p < 0.05$) through treatment in the plant employing a biological nutrient removal (BNR) system. The BNR and secondary clarifier steps in both plants afforded the most removal of ARGs. The relative abundance of ARGs remained unchanged at the AL plant and showed a decreasing trend at the BNR plant. Levels of CBOD₅, nitrate and the human *Bacteroides* fecal marker correlated with ARG concentrations, suggesting these variables may be useful in predicting ARG removal. In conclusion, the effluent coming from the WWTPs contained eight of the studied ARG markers in concentrations ranging from 0.01 to 3.6 log copies/mL, indicating their release into the environment, however, the relative abundance of ARGs was not enriched during treatment in the two WWTPs.

45 **1 Introduction**

46 The overuse and misuse of antibiotics in medical and agricultural settings have
47 resulted in their continuous release into the environment, which has contributed to the
48 development of antibiotic resistant bacteria due to the selective pressure of the antibiotic
49 residues (Davies and Davies, 2010). The efficacy of antibiotics may be reduced if
50 antibiotic resistant genes (ARGs) are transferred to dangerous pathogens. Even in the
51 absence of selective pressures, ARGs can persist in the environment and have been found
52 in naturally occurring bacteria (Allen et al., 2010; Martinez, 2009; Tamminen et al.,
53 2011). However, environments that are influenced by anthropogenic activities show
54 higher concentrations of antibiotic resistant bacteria and ARGs (Allen et al., 2010;
55 Pruden et al., 2006). Internationally, ARGs and antibiotic residues have now been
56 recognized as emerging environmental pollutants (Pruden et al., 2006; Sanderson et al.,
57 2016).

58 The effluent from wastewater treatment plants (WWTPs) is thought to be a major
59 anthropogenic source for the release of antibiotic residues, antibiotic resistant bacteria,
60 and ARGs into the environment (Czekalski et al., 2012; Munir et al., 2011; Rizzo et al.
61 2013). Although WWTPs are designed to remove carbonaceous materials, nutrients and
62 pathogenic bacteria, they are not specifically designed to remove antibiotics and
63 resistance elements (Barancheshme and Munir, 2018). Many ARGs are located on
64 integrons, transposons, or plasmids, which allow mobilization and transfer to other
65 bacteria (Allen et al., 2010). Biological wastewater treatment, due to the presence of large
66 microbial communities, may create an ideal environment for selection and/or horizontal
67 gene transfer of ARGs. Contributing factors include the close proximity of bacteria

68 originating from multiple sources, as well as a selective, nutrient-rich environment
69 containing both antibiotics and heavy metals (Baquero et al., 2008; Davies and Davies,
70 2010; Levy and Marshall, 2004; Rizzo et al., 2013). Therefore, due to the risk of ARG
71 enrichment during the treatment process and subsequent discharge into the environment,
72 understanding wastewater related reservoirs is crucial to assessing the risk of antibiotic
73 resistance transfer in the natural environment.

74 Antibiotics, antibiotic resistant bacteria and ARGs can be affected differently
75 within WWTPs due to variations in the operations of the plant (Batt et al., 2007; Rizzo et
76 al., 2013; Barancheshme and Munir, 2018). Many previous studies have primarily
77 focused on influent and effluent samples, without analyzing the impact of individual
78 steps in wastewater treatment processes (Laht et al., 2014; Narciso-da-rocha et al., 2014;
79 Rodriguez-Mozaz et al., 2015). In order to better design plants to increase removal of
80 resistance determinants and decrease their subsequent release into the environment, it is
81 important to assess the effect of each step in the treatment process. This has therefore
82 been the focus of more recent studies (e.g., Di Cesare et al., 2016, Lee et al., 2017). In
83 Canada, WWTP effluent quality is monitored through measurement of parameters such
84 as total suspended solids (TSS), carbonaceous biological oxygen demand (CBOD₅), or
85 nutrient levels (Wastewater Systems Effluent Regulations, Environment Canada, 2015).
86 Currently, neither antibiotics nor resistance elements are monitored for regulatory
87 compliance purposes. Past studies have found significant correlations between the
88 removal of nutrients and organic matter throughout treatment, and ARG concentrations
89 (Nölvak et al., 2013; Novo and Manaia, 2010). Therefore, examining these correlations
90 may aid in finding potential proxies for monitoring ARG levels in treated wastewater.

91 The objective of this study was to examine how the individual treatment steps in
92 two tertiary WWTPs, using different biological treatment operations, affected the
93 abundance of nine ARGs, and one genetic indicator of mobility potential of ARG
94 cassettes (*int11* integrase). A collection of water quality parameters (*Escherichia coli*,
95 ammonia, phosphorus, CBOD₅, *HF183* human *Bacteroides* marker, bacterial 16S rRNA
96 gene copy numbers, etc.) and presence of selected antibiotics were also assessed in order
97 to examine their potential relationships to ARG abundances.

98

99 **2 Materials and Methods**

100 2.1 Treatment plants and sample collection sites

101 Samples were collected from two different types of tertiary WWTPs in Atlantic
102 Canada; an aerated lagoon (AL plant) system, and a biological nutrient removal (BNR)
103 system (BNR plant). The AL plant was sampled four times (July 7 and 28, and August
104 18, 2015, and February 23, 2016). The BNR plant was sampled on July 12, 2016 and
105 July 18, 2017.

106

107 2.1.1. AL Plant

108 The AL plant discharges approximately 3.8×10^6 L/day of treated effluent into a
109 river. The WWTP services around 600-800 customer connections including a Canadian
110 Forces airbase, which contains a small hospital unit. There would also be several small
111 commercial operations in the sewershed. Treatment steps include screening, two
112 mechanically aerated lagoons (no recycling), two clarifiers, sand filtration, and ultra

113 violet light (UV) disinfection. The hydraulic retention time in the lagoons ranges between
114 18 and 24 hours (personal communications, plant operator), the organic loading rate to
115 the lagoons is 0.12 kg CBOD₅/m²/d, and the mixed liquor suspended solids (MLSS) is
116 approximately 300 mg/L. The surface overflow rate of the clarifiers is 14 m/d. The UV
117 system is a Trojan 3000 PTP (Trojan Technologies, London, ON, Canada) with a design
118 UV dose of 50 mJ/cm². The plant is designed to produce effluent with a maximum of 5
119 mg/L for CBOD₅ and 5 mg/L for TSS. Duplicate water samples were collected in pre-
120 sterilized 1 L Nalgene collection bottles (Thermo Fisher Scientific, Waltham, MA,
121 United States) after the screen (A) [referred to as the influent as it was the first accessible
122 sampling site], after the mechanically aerated lagoons where water from the two lagoons
123 was mixed (B), after the clarifiers where the effluent from two clarifiers was mixed (C),
124 after sand filtration (D) and after UV disinfection (E) as shown in Figure 1A. Water
125 samples were kept on ice while being transported back to the laboratory at Dalhousie
126 University in Halifax, NS (a distance of approximately 150 km), stored at 4°C overnight
127 and processed the following morning.

128

129 2.1.2 BNR Plant

130 The BNR plant services approximately 13,000 people in an urban area, including
131 a local hospital, and light commercial and industrial operations. The effluent discharge of
132 approximately 11.4×10^6 L/day is released into a harbour. The BNR plant uses the
133 modified Johannesburg process, which is designed with activated sludge and sequential
134 anaerobic, anoxic and aerobic reactor zones to remove ammonia, phosphorus, and
135 nitrogen. Treatment steps include primary clarifiers, BNR process reactors, two

secondary clarifiers, and UV disinfection. The hydraulic retention time in the BNR process reactors is approximately 21 hours (personal communication, plant operator). The design MLSS and sludge age in the BNR process reactors are 3500 mg/L and 15 days, respectively. The surface overflow rates of the primary and secondary clarifiers are 30 and 15 m/d, respectively. The UV system is a Wedeco Model TAK55L (Xylem, Rye Brook, NY, USA) operated at a UV dose of 250 mJ/cm². The plant is designed to produce effluent with maximum concentrations of 10 mg/L for CBOD₅, 10 mg/L for TSS, 10 mg/L Total Nitrogen (TN) and 1 mg/L Total Phosphorus (TP). Duplicate samples were collected in pre-sterilized 1 L Nalgene collection bottles (Thermo Fisher Scientific) and collected from the raw influent (A), after the primary clarifiers (B), after BNR treatment (C), after the secondary clarifiers where out-going water from the two clarifiers was mixed (D), and after UV disinfection (E) as shown in Figure 1B. Samples were kept on ice and transported back to the laboratory at Dalhousie University in Halifax, NS (a distance of approximately 300 km) for processing the following morning.

2.2 Analysis of the Abundance of Antibiotic Resistance Genes, Human Fecal Markers and 16S rRNA Gene Copies

2.2.1 Genomic DNA extraction

Microorganisms in wastewater samples (30 to 500 mL) were concentrated either by filtration through 0.45 µm pore size membranes using a vacuum manifold (Millipore, Inc., Bedford, MA) or centrifugation at 3200 × g for 10 minutes. Filters or pellets were stored at -20°C until DNA extraction. Genomic DNA (gDNA) was extracted from the entire filter or cell pellet using the MoBio Powersoil DNA extraction kit (VWR

International, Ville Mont-Royal, QC, Canada) according to the manufacturer's specifications. The concentration and purity of the DNA were evaluated by ultraviolet absorbance spectrophotometry at 260/280 nm and 260/230 nm (Implen Nanophotometer, Implen, München, Germany).

2.2.2. Quantitative real-time PCR

Primer and TaqMan hydrolysis probe sequences and cycling conditions for nine ARGs and one integrase gene were obtained from literature and are listed in Table S1 in the Supplemental Material. The genetic targets included class 1 integrase (*int1*), class A β -lactamase (*bla*_{CTX-M} and *bla*_{TEM}), erythromycin resistance gene (*ermB*), fluoroquinolone resistance gene (*qnrS*), sulphonamide resistance genes (*sul1* and *sul2*), tetracycline resistance gene (*tet(O)*), methicillin resistance gene (*mecA*), and vancomycin resistance gene (*vanA*). These ARGs were chosen to represent a variety of different antibiotic classes and resistance mechanisms as well as clinically relevant genes (Szczepanowski et al., 2009; Volkmann et al., 2004).

The content of bacterial 16S rRNA genes (16S rRNA) was determined by qPCR (reaction conditions described in Table S1) to enumerate the total bacterial community and to allow for the calculation of the relative abundance of ARG relative to the 16S rRNA copy numbers. The human-specific fecal *HF183 Bacteroides* 16S rRNA genetic marker (*HF183*) (Sauer et al., 2011) was also quantified to examine a potential correlation between this DNA marker and ARG abundance. All targets were quantified in all water samples, except for one sampling run (AL plant, July 28, 2015) where *tet(O)*, *mecA*, *vanA* and *HF183* were not tested due to limitations in sample DNA quantities.

181 Control plasmids for *int1*, *bla*_{TEM}, *sul1*, and *sul2* were obtained from Dr. E. Topp
182 (University of Western Ontario, London, ON, Canada) and described in Rahube et al.
183 (2014a). Control plasmids for *bla*_{CTX-M}, *ermB*, *qnrS*, *tet*(O), *mecA*, and *vanA* were
184 described in Neudorf et al. (2017). Concentrations of plasmid DNA were quantified
185 using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific). Standard
186 curves were constructed for each assay using tenfold serial dilutions of plasmid controls
187 and triplicate samples. Quality assurance for standard curves were performed using
188 recommendations from Biorad Real-Time PCR Applications Guide (BioRad, 2013).
189 Efficiencies ranged from 87% to 111% and R^2 values were >0.99 for all standard curves
190 (Table S1). Limits of quantification (LOQ) (copies/reaction) were as follows: *int1*=14.4,
191 *sul1*=11.7, *sul2*=9.6, *tet*(O)=69.0, *ermB*=13.8, *bla*_{CTX-M}=6.2, *bla*_{TEM}=243.0, *qnrS*=112.0,
192 *mecA*=69.0, *vanA*=138.0, 16S rRNA=67000, *HF183*=3630. Limit of detection (LOD)
193 was 5 copies/reaction (or 1 copy/mL for 500 mL sample volumes and 10 copies/mL for
194 50 mL sample volumes).

195 TaqMan qPCR assays for the ARG markers were performed on a Bio-Rad CFX96
196 Touch system (Bio-Rad, Hercules, CA, USA). The following reaction mixture was used:
197 1 x SsoAdvancedTM Universal Probes Supermix (Bio-Rad), 0.9 µM of each primer, 0.25
198 µM TaqMan probe, 2 µL template DNA, and 2 µL of sterile nuclease-free water (Thermo
199 Fisher) to a final volume of 20 µL. A Taqman qPCR assay was also used in
200 quantification of the human faecal *HF183 Bacteroides* marker, where the following
201 reaction mixture was used: 1 x SsoAdvancedTM Universal Probes Supermix (Bio-Rad),
202 0.6 µM of each primer, 0.25 µM TaqMan probe, 2 µL template DNA, and 6.5 µL of
203 sterile nuclease-free water (Thermo Fisher Scientific) to a final volume of 25 µL. SYBR

204 Green qPCR was used to quantify bacterial 16S rRNA gene copy numbers and for this
205 assay, the following reaction mixture was used: 1x Power SYBR[®] Green PCR Master
206 Mix (Applied Biosystems, Foster City, CA, United States), 0.4 μ M primers, and 1 μ L of
207 template DNA, and 7.4 μ L of sterile nuclease-free water (Thermo Fisher Scientific) to a
208 final volume of 20 μ L. Samples and negative controls (no template DNA) were analyzed
209 in duplicate, while standards (control plasmid) reactions were analyzed in triplicates.

210 Raw fluorescence data from the Bio-Rad CFX96 Touch system were imported
211 into the LinRegPCR program (v. 11.4) (Ruijter et al., 2009). For each primer set and
212 qPCR run, the fluorescence threshold was set to obtain a cycle threshold (CT) value for
213 the positive control that matched the concentration specific CT value from the previously
214 established standard curve. The LinRegPCR program automatically calculated the
215 individual sample's CT and E_{fi} (efficiency estimated from fluorescence increase) values.
216 In order to account for differences in efficiencies between samples and standards, a one-
217 point calibration method for absolute quantification was used (Brankatschk et al., 2012).
218 For statistical analyses, any values that fell between the LOD and LOQ were set to $\frac{1}{2}$
219 LOQ value while values that fell below the LOD were set to $\frac{1}{2}$ LOD. For calculation of
220 the relative abundance of genes, the gene copy number of each gene was normalized to
221 the gene copy number of the 16S rRNA gene in each sample and log transformed
222 ($\log(\text{ARG gene copies}/16\text{S rRNA gene copies})$). For calculation of the absolute
223 abundance of genes, gene copies were normalized to the water volume used for gDNA
224 extractions to generate gene copies per mL of water. For the AL plant, the calculation of
225 absolute abundance was done for samples from August and February.

226

227 2.3 Assessment of Water Quality

228 Water samples were analysed within the recommended holding times of 24-48
229 hours for the following water quality parameters: CBOD₅, TSS, volatile suspended solids
230 (VSS), TN, ammonia, nitrate, TP, chemical oxygen demand (COD), total coliforms, and
231 *E. coli*.

232 Analysis of CBOD₅ was performed in duplicate according to the APHA standard
233 method 5210B (American Public Health Association, 2005). TSS and VSS were
234 performed using WhatmanTM 934-AH 47 mm glass fiber filters (Thermo Fisher
235 Scientific) according to APHA standard methods 2540 D (American Public Health
236 Association, 2005). TN was analyzed using Hach[®] TN Test 'N TubesTM (0.5 to 25.0
237 mg/L N, Hach Company, Loveland, CO, United States), according to the manufacturer's
238 procedure. Ammonia and nitrate were measured using high performance ammonia and
239 nitrate electrodes, respectively, as directed by the manufacturer (Thermo Fisher
240 Scientific). The electrodes were attached to an Orion StarTM series meter (Thermo Fisher
241 Scientific). TP was analyzed using Hach[®] TP Test 'N TubeTM ranging from 1.0 to 100.0
242 mg/L PO₄³⁻ (Hach Company), following the manufacturer's procedure. COD was
243 analyzed using Hach[®] COD TNT plus VialTM Test ranging from 20 - 1500 mg/L COD
244 (Hach Company), following the manufacturer's procedure. Total coliforms and *E. coli*
245 were enumerated using IDEXX Colilert[®]-18 and Quanti-Trays[®] (IDEXX Laboratories,
246 Inc., Westbrook, ME, United States) according to the manufacturer's instructions
247 (IDEXX Laboratories, Inc., 2012).

248

249 2.4 Detection of Antibiotics in Water Samples

250 Water samples from the August and February sampling events at the AL plant and
251 from both sampling events at the BNR plant were tested for the content of 10 antibiotics
252 (amoxicillin, cefaclor, cefprozil, cefdinir, levofloxacin, ciprofloxacin, azithromycin,
253 clindamycin, clarithromycin, and triclocarban) at Acadia University (Wolfville, NS,
254 Canada).

255

256 2.4.1 Sample Preparation for Antibiotic Detection

257 The pH of the samples (100 mL) was adjusted to pH 2.5 ± 0.5 with 1 M HCl. The
258 samples were then pumped (5 mL/min) through a vacuum filter apparatus equipped with
259 3 mL Chromabond® HR-X (200 mg sorbent) solid phase extraction columns, which had
260 been pre-conditioned with 6 mL methanol and 6 mL of deionized (DI) water. Sample
261 cartridges were then washed with 3 mL of 10% (v/v) methanol in DI and dried for 5 min
262 under vacuum followed by the elution of analytes with 6 mL of methanol. Eluents were
263 collected and reduced to 1 mL with gentle nitrogen blowdown at 37°C.

264

265 2.4.2 Quantification of Antibiotics

266 Antibiotic analysis was performed using an Agilent 1200 HPLC (Santa Clara,
267 CA, USA) coupled with an Agilent 6410B triple quadrupole mass spectrometer.
268 Chromatographic separation was performed using a 15-cm Agilent Poroshell Eclipse C18
269 column with a 4.6-mm internal diameter and 2.7- μ m particles. The mobile phase
270 consisted of 0.1% (v/v) formic acid buffer. The flow rate of mobile phase was 0.5
271 mL/min. A solvent gradient was programmed to start at 20% (v/v) methanol for 30

seconds, which increased to 100% (v/v) by 20 minutes. The column was held at a constant temperature of 40 °C. Following separation, ionization was conducted with an electrospray ionization source under 35-psi nebulizer pressure. Drying gas temperature was set to 350 °C with a flow rate of 12 L/min. The mass spectrometer was operated in the positive ion mode and the capillary voltage was held at 4000 V. Nebulizing gas and collision gas consisted of 98% nitrogen and ultra-high purity (UHP, 99.999 %) nitrogen, respectively. Precursor-to-product ion transitions were established for all target antibiotics. To boost the sensitivity of analysis, only one transition was monitored for each compound. Fragmentor voltage and collision energy were carefully optimized to achieve maximum response for each transition. The mass spectrometer parameters and transitions are shown in the supplementary information Table S2.

2.4.3 Quality assurance and quality control

To assist with quality assurance and quality control, solvent blank samples were included. Sampling bottles were pre-cleaned by manufacturers. These bottles and other glassware were thoroughly cleaned using DI water and methanol. Salinization of glassware was not performed because spiked samples did not show loss of target compounds.

Laboratory control samples were prepared by spiking a 100 mL DI sample containing 5 g of sodium chloride with an antibiotic mixture and subjecting the sample to the same analytical method. The recoveries of the target antibiotics were analyzed and reported. An internal standard was used to ensure the quality of instrumental analysis and

294 sample preparation. Duplicates were included to verify the reproducibility of the
295 analytical method.

296

297 2.5 Data Analysis

298 One-way analysis of variance (ANOVA), Tukey HSD test, and t-tests were used
299 to assess statistically significant differences ($p < 0.05$) among samples. Log reductions
300 were calculated as the log difference between treatment steps or influent/effluent. Pearson
301 correlation coefficients were calculated (after performing check of normality) to assess if
302 significant ($p < 0.05$) correlations existed between the total ARG marker concentrations
303 and water quality parameters. Analysis was performed using GraphPad Prism version
304 7.00 for Mac OS X (GraphPad Software, La Jolla California USA, www.graphpad.com),
305 and Microsoft Excel, 2016. The total ARG marker concentrations were calculated as the
306 average of the sum of all tested ARG marker concentrations in the samples for each
307 individual sample site and run.

308

309 3 Results and Discussion

310

311 3.1 The absolute abundance of ARGs during treatment in the AL and BNR plants

312 Measurements of water quality parameters and content of ARGs revealed no
313 significant differences ($p > 0.05$) between sample runs, indicating consistent performance
314 of the WWTPs. For further analysis, the averages of the results from all sample runs are
315 presented.

316 All ARG gene targets, with the exception of *mecA* (only intermittent detection)
317 and *vanA* (never detected above LOD), were consistently detected in the municipal
318 wastewater arriving at both WWTPs (Figures 2 and 3). Other WWTP studies have
319 reported detection of *mecA* and *vanA*, which confer resistance to methicillin and
320 vancomycin, respectively, both of which are antibiotics of last resort (Narciso-da-rocha et
321 al., 2014; Szczepanowski et al., 2009; Volkmann et al., 2004). The low levels or absence
322 of these targets in this study may be due to the fact that these WWTPs were servicing
323 smaller urban centres (< 15,000 people). Furthermore, *mecA* and *vanA* genes can be
324 present on chromosomal DNA as opposed to plasmids (Biavasco et al., 2007; Cetinkaya
325 et al., 2000; Colomer-Lluch et al., 2011; Katayama et al., 2000) resulting in a limited
326 range of host bacteria with lower presence/survival in the WWTP environment
327 (Goldstein et al., 2012). ARGs belonging to the *tet* and *sul* families were detected in all
328 influent samples and represent resistance genes to some of the oldest and most commonly
329 consumed antibiotics (Al-Jassim et al., 2015; Auerbach et al., 2007; Du et al., 2015; Laht
330 et al., 2014; LaPara et al., 2011). Other less studied ARGs (*qnrS*, *ermB*, *bla*_{CTX-M}, *bla*_{TEM})
331 have also been previously detected in wastewater samples, and represent genes that are
332 often plasmid-borne and confer resistance to the latest generations of antibiotics (Marti et
333 al., 2013; Narciso-da-Rocha et al., 2014; Rodriguez-Mozaz et al., 2014).

334 Overall, the AL treatment continuum led to a significant ($p < 0.05$) decrease in the
335 total ARG marker abundance of 1.77 log copies/mL from the influent sewage (total ARG
336 of 6.29 log copies/mL) to the treated effluent (total ARG of 4.52 log copies/mL (Figure
337 2A). Total ARG concentrations decreased by the following log copies/mL after each
338 treatment step: 0.16 (AL), 1.54 (secondary clarifier), 0.26 (sand filters), -0.20 (UV). This

339 showed that the secondary clarifiers were responsible for the largest incremental removal
340 of ARGs, an observation agreeing with the result from the BNR plant (see below) and
341 WWTP2 in the study of Lee et al. (2017). This removal may likely have occurred through
342 the sedimentation processes and may imply that the generated biosolids contain a large
343 amount of the ARGs, as has previously been observed (Börjesson et al., 2010; Chen and
344 Zhang, 2013; Mao et al., 2015; Nnadozie et al., 2018). The AL, sand filters and UV
345 treatment steps contributed little to the removal of ARGs in this WWTP.

346 In the AL plant, the content of individual genes exhibited, with the exception of
347 *sul1* and *bla*_{CTX-M}, significant ($p < 0.05$) decreases from 0.94 log copies/mL (*mecA*) to
348 2.23 log copies/mL (*qnrS*), when comparing the influent and effluent water samples
349 (Figure 2A). Previous research has suggested that sulfamethoxazole-resistant bacteria are
350 difficult to remove (Gao et al., 2012), which may explain the insignificant reduction of
351 *sul1*. The significant ($p < 0.05$) 1.74 log copies/mL decrease in the AL plant's absolute
352 abundance of 16S rRNA gene from 9.23 log copies/mL in the influent to 7.49 log
353 copies/mL in the effluent (Figure 2C) was similar to the decrease in the total ARG
354 concentration. Individual ARG concentration levels ranged from 3.27 (*mecA*) to 5.85
355 (*qnrS*) and from 2.32 (*mecA*) to 4.15 (*int11*) log copies/mL in the influent and effluent
356 samples, respectively.

357 In the BNR plant, there was a significant ($p < 0.05$) decrease in total ARG
358 concentration of 2.69 log copies/mL, from 7.01 in the raw influent to 4.33 log copies/mL
359 in the effluent (Figure 3A). After the individual treatment steps, the total ARG
360 concentration decreased by the following log copies/mL: 0.15 (primary clarifier), 1.36
361 (BNR), 0.62 (secondary clarifiers), 0.56 (UV). This means that in comparison to the AL

362 plant, the total ARG removal in the BNR plant was 0.92 log copies/mL or 88% higher
363 (2.69 vs. 1.77 log copies/mL). Moreover, the BNR treatment step was responsible for the
364 largest incremental removal of ARGs in the wastewater system; this was in contrast to the
365 minimal removal observed in the biological treatment unit (i.e., the mechanically aerated
366 lagoons) of the AL plant. The finding that the BNR plant's sequential use of anaerobic,
367 anoxic and aerobic reactors improved the removal of ARGs concurs with reports by
368 Munir et al. (2011), Du et al. (2015) and Lee et al. (2017), who studied WWTPs with
369 similar combinations of reactors with different oxygen conditions. Both the secondary
370 clarification and the UV treatment in the BNR plant contributed significantly ($p < 0.05$) to
371 the removal of ARGs. The UV doses were 50 and 250 mJ/cm^2 in the AL and BNR plants,
372 respectively, which likely explained the larger ARG log removal of 0.56 after this step in
373 the BNR plant as compared to no decrease at the AL plant. In agreement with our
374 observations, Lee et al. (2017) reported no decrease in ARGs following UV disinfection
375 at a dosage of 27 mJ/cm^2 in two different WWTPs, while Zhang et al. (2015) reported
376 similar log reductions for various ARGs after a UV dose of 250 mJ/cm^2 .

377 Decreases in individual ARG genes from influent to effluent ranged from 2.20
378 (*mecA*) to 3.26 (*bla*_{CTX-M}) log copies/mL. Interestingly, Helt (2012) and Börjesson et al.
379 (2010) observed lower log removals of between 1.26 and 1.62 log copies/mL in a pilot
380 and full scale BNR system, respectively, which were removal levels similar to those
381 reported for the AL plant in the present study. The absolute abundance of the 16S rRNA
382 gene decreased significantly ($p < 0.05$) by 1.84 log copies/mL from 9.36 to 7.52 log
383 copies/mL in the influent and effluent, respectively (Figure 2C). This indicated a lower
384 relative reduction of the total bacterial population than of the ARGs, whose concentration

385 levels (log copies/mL) ranged from 2.21 (*mecA*) to 6.65 (*int11*) in the influent and from
386 0.01 (*mecA*) to 4.09 (*int11*) in the effluent.

387

388 3.2 Relative abundance of ARGs at the AL and BNR plants

389 At the AL plant, no significant differences ($p>0.05$) were observed in the relative
390 abundance of the total ARG and the individual ARG markers between wastewater
391 treatment steps, except for *sul2*, whose relative abundance significantly ($p<0.05$)
392 increased after the lagoon step (Figure 2B). Hence, there was neither a relative
393 enrichment nor reduction in the relative gene abundances through the AL treatment train,
394 meaning that the total bacterial community (here represented by 16S rRNA gene copies)
395 and antibiotic resistant bacteria were being reduced at comparable rates. As such it
396 appeared that the AL plant neither selected for or against bacteria harbouring ARGs. This
397 has, to the best of our knowledge, not previously been shown for AL type WWTPs.

398 Similar to the results from the AL plant, results from the BNR plant showed that
399 the relative total ARG abundances remained unchanged ($p>0.05$) through the different
400 treatment steps (Figure 3B). However, when examining each gene individually, the
401 relative abundance of most genes tended to decrease after the BNR step (significant
402 ($p<0.05$) for *bla*_{CTX-M}), with the exception of *sul2* and *mecA*, which increased slightly
403 (Figure 3B) (also observed for *sul2* at the AL plant). These results indicate that the BNR
404 biological treatment may more efficiently decrease bacteria carrying ARGs than the AL
405 treatment. It may be that the processes designed to remove nutrients may not favour
406 bacteria harbouring the surveyed ARGs leading to the lower abundances of these
407 bacterial communities after BNR treatment. The literature is still conflicting on whether

the relative abundance is changed in WWTPs as Mao et al. (2015) observed increases in the relative ARG abundance in two Chinese WWTPs while Di Cesare et al. (2016) and Bengtson-Palme et al. (2016) saw the opposite trend or no change in water samples from three Italian and three Swedish WWTPs, respectively. Besides varying operational parameters which make direct comparisons difficult, the nature of the raw wastewater may also be important. This was indicated in the study of two WWTPs using a series of anoxic, anaerobic and aerobic reactors as well as sedimentation, coagulation, filtration and UV, where the WWTP receiving predominantly municipal and industrial wastewater reported reduction in the relative abundance of several ARGs (*tet*, *sul*, *erm*, *qnr*, *bla*) while the relative abundance was enriched for *sul*, *qnr* and *bla* in the WWTP receiving a higher load of agricultural wastewater (Lee et al. 2017).

At both plants, *int1* became the most detected gene in the effluent (Figures 2A, B and 3A, B). This was also observed by Narciso-da-Rocha et al. (2014), who suggested that *int1* may be stable in wastewater. The detection of the gene marker for class I integrons in wastewater may provide a global perspective on antibiotic resistance and ARGs as integrons have the ability to carry and allow the transfer of multiple ARGs (Rizzo et al., 2013; Rahube et al., 2014b).

3.3 Changes in *E. coli* and HF183 human *Bacteroides* marker concentrations

Treatment at both plants significantly ($p < 0.05$) decreased average *E. coli* counts from 4.7-5.3 log MPN/mL in the influent to effluent values of < 0.01 MPN/ mL (Figures 2C and 3C). The human *Bacteroides* fecal HF183 marker also decreased significantly ($p < 0.05$) at both plants, with removals of 2.2 and 2.6 log copies/mL from the influent to

431 effluent water samples at the AL and BNR plants, respectively. Removal of *E. coli* and
432 the *HF183* marker at the AL plant showed no correlation to the total ARG marker
433 concentrations ($p>0.05$), indicating no link between the removal of antibiotic resistance
434 related genes and *E. coli* or the *HF183* marker. This was contrasted by the data from the
435 BNR plant, where the Pearson correlation coefficient ($r=0.97$) for the human *HF183*
436 marker in relation to total ARG marker concentrations was significant ($p<0.05$),
437 indicating that removal of the selected gene markers of antibiotic resistance and the
438 marker of human fecal contamination followed the same trend. Future research is
439 recommended to investigate the correlations between ARGs and *E. coli*/molecular fecal
440 markers in other WWTPs to assess the mechanisms and relevance of these indicators for
441 prediction of ARG removal.

442 Treatment in both the AL and BNR plants caused a 6 log MPN/mL decrease in
443 viable *E. coli* counts but only around 2-3 log decreases for ARGs, 16S rRNA and *HF183*
444 gene copies/mL. The use of qPCR for detection of genetic elements, such as ARGs and
445 *HF183*, to monitor removal or inactivation of fecal indicator bacteria in wastewater may
446 be problematic as the decrease in genetic markers may not follow decreases in viable
447 bacteria levels due to possible detection of markers from dead cells or cell-free DNA
448 (Chern et al., 2014; Zhang et al., 2018). This phenomenon is likely observed in Figures 2
449 and 3, where the UV disinfection step significantly reduced culturable *E. coli* counts,
450 while ARG, 16S rRNA and *HF183* copies/mL remained unchanged. However,
451 exogenous DNA can still be transferred to other bacteria through transformation (Frost et
452 al., 2005), especially while in presence of selective pressures (e.g., antibiotics and

metals), therefore extracellular ARGs entering the environment through effluent discharge may still present a possible threat to public health.

3.4 Water quality assessment and correlation to gene abundance

The AL plant was efficient at nutrient and solids removal as all measured parameters decreased between 0.5 and 2 orders of magnitude (Table 1), with the exception of nitrate which was increased due to conversion of ammonia into nitrate (nitrification). The plant met the regulatory targets of 5 mg/L for both TSS and CBOD₅ in the effluent. In regard to correlations between total ARG concentrations and water quality at the tested sites, nitrate ($r=-0.92$, $p<0.05$) and CBOD₅ ($r=0.95$, $p<0.05$) showed significant correlations (Table 1), suggesting that decreasing CBOD₅ and increasing nitrate levels may help predict a decrease in ARGs in wastewater effluent from this type of plant design.

The BNR plant also met its regulatory targets of reducing TSS and CBOD₅ to below 25 mg/L with its efficient removal of nutrients and solids (Table 2). While there was a trend toward COD, CBOD₅ and ammonia correlating with ARG removal, these relationships were not statistically significant ($p>0.05$).

Relationships between removal of nutrients and ARG abundance have been suggested in other studies (Börjesson et al., 2010; Du et al., 2015; Nölvak et al., 2013; Novo and Manaia, 2010). Similar to the AL plant, Nölvak et al. (2013) found significant correlations between nitrate (negative) and organic matter (positive) with ARG concentrations. Further testing would be needed for the BNR plant to determine if the trends observed would become significant.

476

477 3.5 Antibiotic Results and Correlations

478 All tested antibiotics, besides cefprozil and cefaclor, were detected in various
479 samples from both plants (Supplemental material Tables S3 and S4). Of the tested
480 penicillin antibiotics, only amoxicillin and cefdinir were detected despite their high
481 consumption, which may be due to their chemical instability (Graham et al., 2011). The
482 tested macrolides (azithromycin, clindamycin and clarithromycin) were detected in
483 samples obtained after all treatment steps indicating their poor removal in both WWTPs
484 as was also observed by Marx et al. (2015) and Bengtson-Palme et al. (2016).
485 Interestingly, both influent and effluent water samples from the AL plant showed higher
486 concentrations of antibiotics compared to the BNR plant, a fact which warrants further
487 investigations and may have contributed to the lower ARG removals in the AL plant.
488 Correlation analysis was performed and showed no significant ($p < 0.05$) relationships
489 between the individual ARG absolute concentrations and the antibiotics they confer
490 resistance to (see Table S2). Other studies have found correlations in the past (Gao et al.,
491 2012; Gao et al., 2015; Rodriguez-Mozaz et al., 2015), while Bengtson-Palme et al.
492 (2016) found none. Possible correlations may depend on the class of antibiotics since
493 their transport and/or susceptibility to degradation vary markedly (Marx et al., 2015;
494 Nnadozie et al., 2017).

495

496 **4 Conclusions**

497 The tertiary WWTPs reduced effluent ARG marker concentrations by approximately 2
498 log copy/mL but were still releasing total ARG markers in the order of 1.3×10^{14} and 2.8
499 $\times 10^{14}$ copies/day for the AL and BNR plant, respectively. The best removal was obtained
500 in the BNR reactors (modified Johannesburg process), which uses a series of reactors
501 with anoxic, anaerobic and aerobic reactors, as opposed to the aerobic AL lagoons. The
502 secondary clarifiers effected significant removal of ARGs in both WWTPs and did a high
503 UV dose of 250 mJ/cm^2 in the BNR plant. The relative abundance of ARGs generally
504 remained unchanged in samples taken along the entire treatment train of the AL and BNR
505 plant, indicating that these types of wastewater treatment systems do not promote the
506 enrichment of ARGs in the bacterial population. Changes in organic matter and the
507 removal of the human fecal *HF183* marker may be useful in predicting ARG removal,
508 however, further testing is needed to solidify these relationships.

509

510 **5 Acknowledgments**

511 We would like to thank the local authorities for allowing us access to the two
512 wastewater treatment plants. We would also like to thank our team members for their
513 assistance in the field and laboratory (Audrey Hiscock, Robert Johnson, Amy Jackson,
514 and Joanna Poltarowicz). Funding for this project was provided by the Natural Science
515 and Engineering Research Council of Canada (STPGP 463352 – 14).

References

- Al-Jassim, N., Ansari, M.I., Harb, M., Hong, P.-Y., 2015. Removal of bacterial contaminants and antibiotic resistance genes by conventional wastewater treatment processes in Saudi Arabia: Is the treated wastewater safe to reuse for agricultural irrigation? *Water Res.* 73, 277–290. doi:10.1016/j.watres.2015.01.036
- Allen, H.K., Donato, J., Wang, H.H., Cloud-Hansen, K. A, Davies, J., Handelsman, J., 2010. Call of the wild: antibiotic resistance genes in natural environments. *Nat. Rev. Microbiol.* 8, 251–259. doi:10.1038/nrmicro2312
- American Public Health Association (APHA), 2005. Standard methods for the examination of water and waste water. 21st ed. Washington, DC: American Public Health Association
- Auerbach, E.A., Seyfried, E.E., McMahon, K.D., 2007. Tetracycline resistance genes in activated sludge wastewater treatment plants. *Water Res.* 41, 1143–1151. doi:10.1016/j.watres.2006.11.045
- Barancheshme, F., Munir, M. 2018. Strategies to combat antibiotic resistance in the wastewater plants. *Front. Microbiol.* 8, 2603. doi:10.3389/fmicb.2017.02603
- Baquero, F., Martínez, J.L., Cantón, R., 2008. Antibiotics and antibiotic resistance in water environments. *Curr. Opin. Biotechnol.* 19, 260–265. doi:10.1016/j.copbio.2008.05.006
- Batt, A.L., Kim, S., Aga, D.S., 2007. Comparison of the occurrence of antibiotics in four full-scale wastewater treatment plants with varying designs and operations. *Chemosphere* 68, 428–435. doi:10.1016/j.chemosphere.2007.01.008
- Bengtson-Palme, J., Hammerén, R., Pal, C., Östman, M., Björnlenius, B., Flach, C-F., Fick, J., Tysklind, M., Larsson, D.G.J., 2016. Elucidating selection processes for antibiotic resistance in sewage treatment plants using metagenomics. *Sci. Tot. Environ.* 572, 697-712. <http://dx.doi.org/10.1016/j.scitotenv.2016.06.228>
- Biavasco, F., Foglia, G., Paoletti, C., Zandri G., Magi, G., Guaglianone, E., Sundsfjord, A., Pruzzo, C., Donelli, G., Facinelli, B., 2007. *VanA*-type enterococci from humans, animals, and food: species distribution, population structure, Tn1546 typing and location, and virulence determinants. *Appl. Environ. Microbiol.* 73, 3307–3319.
- Bio-Rad, 2013. CFX384 / CFX96 Touch TM Real-Time PCR Detection Systems

558 Instruction Manual, Manual.
559

560 Börjesson, S., Mattsson, A., Lindgren, P.E., 2010. Genes encoding tetracycline resistance
561 in a full-scale municipal wastewater treatment plant investigated during one year. *J.*
562 *Water Health*, 8, 247–256. doi:10.2166/wh.2009.159
563

564 Brankatschk, R., Bodenhausen, N., Zeyer, J., Burgmann, H., 2012. Simple absolute
565 quantification method correcting for quantitative PCR efficiency variations for
566 microbial community samples. *Appl. Environ. Microbiol.* 78, 4481–4489.
567 doi:10.1128/AEM.07878-11
568

569 Cetinkaya, Y., Falk, P., Mayhall, C.G., 2000. Vancomycin-resistant Enterococci. *Clin.*
570 *Microbiol. Rev.* 13(4), 686-707.
571

572 Chen, H., Zhang, M., 2013. Effects of advanced treatment systems on the removal of
573 antibiotic resistance genes in wastewater treatment plants from Hangzhou, China.
574 *Environ. Sci. Technol.* 47, 8157–8163.
575

576 Chern, E.C., Brenner, K., Wymer, L., Haugland, R.A., 2014. Influence of wastewater
577 disinfection on densities of culturable fecal indicator bacteria and genetic markers. *J*
578 *Water Health*. 12(3), 410-417.
579

580 Colomer-Lluch, M., Jofre, J., Muniesa, M., 2011. Antibiotic resistance genes in the
581 bacteriophage DNA fraction of environmental samples. *PLoS ONE*. 6(3), 1-11.
582

583 Czekalski, N., Berthold, T., Caucci, S., Egli, A., Bürgmann, H., 2012. Increased levels of
584 multiresistant bacteria and resistance genes after wastewater treatment and their
585 dissemination into Lake Geneva, Switzerland. *Front. Microbiol.* 3:106. [http://dx.doi.](http://dx.doi.org/10.3389/fmicb.2012.00106)
586 [org/10.3389/fmicb.2012.00106](http://dx.doi.org/10.3389/fmicb.2012.00106).
587

588 Davies, J., Davies, D., 2010. Origins and evolution of antibiotic resistance. *Microbiol.*
589 *Mol. Biol. Rev.* 74, 417–433. doi:10.1128/MMBR.00016-10
590

591 Di Cesare, A., Eckert, E.M., D'Urso, S., Bertoni, R., Gillan, D., Wattiez, R., Corno, G.,
592 2016. Co-occurrence of integrase 1, antibiotic and heavy metal resistance genes in
593 municipal wastewater treatment plant. *Water Res.* 94, 208-214.
594 <http://dx.doi.org/10.1016/j.watres.2016.02.049>
595

596 Du, J., Geng, J., Ren, H., Ding, L., Xu, K., Zhang, Y., 2015. Variation of antibiotic
597 resistance genes in municipal wastewater treatment plant with A2O-MBR system.
598 *Environ. Sci. Pollut. Res.* 22, 3715–3726. doi:10.1007/s11356-014-3552-x
599

Environment Canada, 2015. Wastewater Systems Effluent Regulations.
<http://www.ec.gc.ca/eu-ww/>. Accessed 14 June 2018.

Frost, L.S., Leplae, R., Summers, A.O., Toussaint, A., 2005. Mobile genetic elements: the agents of open source evolution. *Nat. Rev. Microbiol.* 3, 722–732.
doi:10.1038/nrmicro1235

Gao, P., He, S., Huang, S., Li, K., Liu, Z., Xue, G., Sun, W., 2015. Impacts of coexisting antibiotics, antibacterial residues, and heavy metals on the occurrence of erythromycin resistance genes in urban wastewater. *Appl. Microbiol. Biotechnol.* 99, 3971–3980. doi:10.1007/s00253-015-6404-9

Gao, P., Munir, M., Xagorarakis, I., 2012. Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. *Sci. Total Environ.* 421–422, 173–183. doi:10.1016/j.scitotenv.2012.01.061

Graham DW, Olivares-Rieumont S, Knapp CW, Lima L, Werner D, et al., 2011. Antibiotic resistance gene abundances associated with waste discharges to the Almendares River near Havana, Cuba. *Environ Sci Technol.* 45, 418–424.

Goldstein, R.E.R., Micallef, S., Gibbs, S., Davis, J., George, A., Kleinfelter, L., et al., 2012. Methicillin-resistant *Staphylococcus aureus* detected at four U.S. wastewater treatment plants. *Environ. Health Perspect.* 120, 1551–1558.

Helt, C., 2012. Occurrence, fate, and mobility of antibiotic resistant bacteria and antibiotic resistance genes among microbial communities exposed to alternative wastewater treatment systems. (Doctoral thesis). University of Waterloo, Ontario, Canada.

Katayama, Y., Ito, T., Hiramatsu, K., 2000. A new class of genetic element, *Staphylococcus* cassette chromosome *mec*, encodes methicillin resistance in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 44(6), 1549–1555.

Laht, M., Karkman, A., Voolaid, V., Ritz, C., Tenson, T., Virta, M., Kisand, V., 2014. Abundances of tetracycline, sulphonamide and beta-lactam antibiotic resistance genes in conventional wastewater treatment plants (WWTPs) with different waste load. *PLoS One*, 9, 1–8. doi:10.1371/journal.pone.0103705

LaPara, T.M., Burch, T.R., Mcnamara, P.J., Tan, D.T., Yan, M., Eichmiller, J.J., 2011. Tertiary-treated municipal wastewater is a significant point source of antibiotic resistance genes into Duluth-Superior Harbor. *Environ. Sci. Technol.* 45, 9543–9549.

643
644 Lee, J., Jeon, J.H., Shin, J., Jang, H.M., Kim, S., Son, M.S., Kim, Y.M. 2017.
645 Quantitative and qualitative changes in antibiotic resistance genes after passing
646 through treatment processes in municipal wastewater treatment plants. *Sci. Total*
647 *Environ.* 605-606, 906-914.
648
649 Levy, S.B., Marshall, B., 2004. Antibacterial resistance worldwide: causes, challenges
650 and responses. *Nat. Med.* 10, S122–S129. doi:10.1038/nm1145
651
652 Mao, D., Yu, S., Rysz, M., Luo, Y., Yang, F., Li, F., Hou, J., Mu, Q, Alvarez, P.J.J.
653 2015. Prevalence and proliferation of antibiotic resistance genes in two municipal
654 wastewater treatment plants. *Water Res.* 85, 458-466.
655
656 Marti, E., Jofre, J., Balcazar, J.L., 2013. Prevalence of antibiotic resistance genes and
657 bacterial community composition in a river influenced by a wastewater treatment
658 plant. *PLoS One*, 8, 1–8.
659
660 Martinez, J.L., 2009. Environmental pollution by antibiotics and by antibiotic resistance
661 determinants. *Environ. Pollut.* 157, 2893–2902. doi:10.1016/j.envpol.2009.05.051
662
663 Marx, C., Günther, N., Schubert, S., Oertel, R., Ahnert, M., Krebs, P., Kuehn, V. 2015.
664 Mass flow of antibiotics in a wastewater treatment plant focusing on removal
665 variation due to operational parameters. *Sci. Total Environ.* 538, 779-788.
666 <http://dx.doi.org/10.1016/j.scitotenv.2015.08.112>
667
668 Munir, M., Wong, K., Xagorarakis, I., 2011. Release of antibiotic resistant bacteria and
669 genes in the effluent and biosolids of five wastewater utilities in Michigan. *Water*
670 *Res.* 45, 681–693. doi: 10.1016/j.watres.2010.08.033
671
672 Narciso-da-Rocha, C., Varela, A.R., Schwartz, T., Nunes, O.C., Manaia, C.M., 2014.
673 Resistance *bla*_{TEM} and *vanA* as indicator genes of antibiotic resistance contamination
674 in a hospital – urban wastewater treatment plant system. *J. Glob. Antimicrob. Resist.*
675 2, 309–315. doi:10.1016/j.jgar.2014.10.001.
676
677 Neudorf, K., Huang, Y.N., Ragush, C., Yost, C., Jamieson, R., Truelstrup Hansen, L.,
678 2017. Antibiotic resistance genes in municipal wastewater treatment systems
679 and receiving waters in Arctic Canada. *Sci. Total Environ.* 598, 1085-1094.
680 doi:10.1016/j.scitotenv.2017.04.151
681
682 Nnadozie, C.F., Kumari, S., Bux, F. 2017. Status of pathogens, antibiotic resistance genes
683 and antibiotic residues in wastewater treatment systems. *Rev. Environ. Sci.*
684 *Biotechnol.* 16, 491-515. doi: 10.1007/s11157-017-9438-x

685
686 Nõlvak, H., Truu, M., Tiirik, K., Oopkaup, K., Sildvee, T., Kaasik, A., Mander, Ü., Truu,
687 J., 2013. Dynamics of antibiotic resistance genes and their relationships with system
688 treatment efficiency in a horizontal subsurface flow constructed wetland. *Sci. Total*
689 *Environ.* 461–462, 636–644. doi:10.1016/j.scitotenv.2013.05.052
690
691 Novo, A., Manaia, C.M., 2010. Factors influencing antibiotic resistance burden in
692 municipal wastewater treatment plants. *Appl. Microbiol. Biotechnol.* 87, 1157–
693 1166. doi:10.1007/s00253-010-2583-6
694
695 Pruden, A., Pei, R., Storteboom, H., Carlson, K.H., 2006. Antibiotic resistance genes as
696 emerging contaminants: Studies in northern Colorado. *Environ. Sci. Technol.* 40,
697 7445–7450. doi:10.1021/es060413l
698
699
700 Rahube, T.O., Marti, R., Scott, A., Tien, Y., Murray, R., Sabourin, L., Zhang, Y., Duenk,
701 P., Lapen, D.R., Topp, E., 2014a. Impact of fertilizing with raw or anaerobically
702 digested sewage sludge on the abundance of antibiotic-resistant coliforms, antibiotic
703 resistant genes, and pathogenic bacteria in soil and on vegetables at harvest. *Appl.*
704 *Environ. Microbiol.* 80, 6898–6907.
705
706 Rahube, T.O., Viana, L.S., Koraimann, G., Yost, C.K., 2014b. Characterization and
707 comparative analysis of antibiotic resistance plasmids isolated from a wastewater
708 treatment plant. *Front Microbiol* 5, 558. doi:10.3389/fmicb.2014.00558
709
710 Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., Fatta-
711 Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for antibiotic
712 resistant bacteria and genes spread into the environment: a review. *Sci. Total*
713 *Environ.* 447, 345–60. doi:10.1016/j.scitotenv.2013.01.032
714
715 Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sànchez-Melsió, A.,
716 Borrego, C.M., Barceló, D., Balcázar, J.L., 2015. Occurrence of antibiotics and
717 antibiotic resistance genes in hospital and urban wastewaters and their impact on the
718 receiving river. *Water Res.* 69, 234–242. doi:10.1016/j.watres.2014.11.021
719
720 Ruijter, J.M., Ramakers, C., Hoogaars, W.M.H., Karlen, Y., Bakker, O., Van Den Hoff,
721 M.J.B., Moorman, A.F.M., 2009. Amplification efficiency: Linking baseline and
722 bias in the analysis of quantitative PCR data. *Nucleic Acids Res.* 37.
723 doi:10.1093/nar/gkp045
724
725 Sanderson, H., Fricker, C., Brown, R.S., Majury, A., Liss, S.N., 2016. Antibiotic
726 resistance genes as an emerging environmental contaminant. *Environ. Rev.* 218, 27–
727 39. doi:10.1139/er-2015-0069

728
729 Sauer, E.P., VandeWalle, J.L., Bootsma, M.J., McLellan, S.L., 2011. Detection of the
730 human specific *Bacteroides* genetic marker provides evidence of widespread sewage
731 contamination of stormwater in the urban environment. *Water Res.* 45, 4081–4091.
732 doi:10.1016/j.watres.2011.04.049
733
734 Szczepanowski, R., Linke, B., Krahn, I., Gartemann, K.H., Gützkow, T., Eichler, W.,
735 Pühler, A., Schlüter, A., 2009. Detection of 140 clinically relevant antibiotic-
736 resistance genes in the plasmid metagenome of wastewater treatment plant bacteria
737 showing reduced susceptibility to selected antibiotics. *Microbiology.* 155, 2306–
738 2319. doi:10.1099/mic.0.028233-0
739
740 Tamminen, M., Karkman, A., Lohmus, A., Muziasari, W.I., Takasu, H., Wada, S.,
741 Suzuki, S., Virta, M., 2011. Tetracycline resistance genes persist at aquaculture
742 farms in the absence of selection pressure tetracycline resistance genes persist at
743 aquaculture farms in the absence of selection pressure. *Environ. Sci. Technol.* 45,
744 386–391. doi:10.1021/es102725n
745
746 Volkmann, H., Schwartz, T., Bischoff, P., Kirchen, S., Obst, U., 2004. Detection of
747 clinically relevant antibiotic-resistance genes in municipal wastewater using real-
748 time PCR (TaqMan). *J. Microbiol. Methods* 56, 277–286.
749 doi:10.1016/j.mimet.2003.10.014
750
751 Zhang, Y., Zhuang, Y., Geng, J., Ren, H., Zhang, Y., Ding, L., Xu, K., 2015. Inactivation
752 of antibiotic resistance genes in municipal wastewater effluent by chlorination and
753 sequential UV/chlorination disinfection. *Sci. Total Environ.* 512-513, 125-132.
754
755 Zhang, Y., Li, A., Dai, T., Li, F., Xie, H., Chen, L., Wen, D., 2018. Cell-free DNA: A
756 neglected source for antibiotic resistance genes spreading from WWTPs. *Env.*
757 *Sci. Technol.* 52, 248-257. doi:10.1021/acs.est.7b04283
758

759 **Figure Legends.**

760

761 Figure 1. Schematic layout of the treatment trains of A) the aerated lagoon (AL) plant
762 and B) the biological nutrient removal (BNR) plant with sampling sites indicated by
763 letters (A-E).

764

765 Figure 2. Effect of individual treatment steps in an mechanically aerated lagoon
766 wastewater treatment plant on the absolute and relative abundances of ARGs and fecal
767 indicators in water samples (averages $\pm$ standard deviations). a) Absolute abundances of
768 each ARG, *int11* and the sum of all ARG markers (Total ARG) (n=4). b) Relative
769 abundances of each ARG, *int11* and Total ARG (n=8). c) Concentration of 16S rRNA
770 genes, *E. coli* and the human *HF183 Bacteroides* marker (n=4). (Sand is an abbreviation
771 for the sand filter)

772

773 Figure 3. Effect of individual treatment steps in a biological nutrient removal reactor
774 wastewater treatment plant on the absolute and relative abundances of ARGs and fecal
775 indicators in water samples (n=4, averages $\pm$ standard deviation). a) Absolute abundances
776 of each ARG, *intl1* and the sum of all ARG markers (Total ARG). b) Relative
777 abundances of each ARG, *intl1* and Total ARG. c) Concentration of 16S rRNA gene
778 copies, *E. coli* and the human *HF183 Bacteroides* marker. (1st and 2nd refer to the
779 primary and secondary clarifiers).
780

Tables.

Table 1. AL plant water quality data and Pearson correlation coefficients (average results ± standard deviations from four sampling events).

	Nitrate (mg/L)	Ammonia (mg/L)	TN (mg/L)	TSS (mg/L)	VSS (mg/L)	COD (mg/L)	TP (mg/L)	CBOD ₅ (mg/L)
Influent	1.5 ±1.7	18.8 ± 5.8	31.3 ± 6.76	160.9 ± 65.3	151.2 ± 59.6	406.1 ± 70.3	9.4 ± 2.1	169.3 ± 42.2
Lagoon	5.5 ± 2.7	1.0 ± 0.55	201.1 ± 41.83	2861.3 ± 1006.0	2332.0 ± 812.5	4272.3 ± 1478.0	255.6 ± 69.0	242.3 ± 92.3
Clarifier	8.1 ± 3.7	0.7 ± 0.61	10.5 ± 7.57	5.9 ± 1.9	4.8 ± 1.2	21.9 ± 5.4	3.3 ± 3.7	3.6 ± 3.3
Sand Filter	8.6 ± 3.6	0.3 ± 0.40	7.5 ± 5.00	1.6 ± 1.2	1.4 ± 0.66	21.3 ± 0.50	3.1 ± 3.7	2.9 ± 1.3
UV	8.3 ± 3.4	0.3 ± 0.39	6.7 ± 4.00	1.6 ± 0.66	1.7 ± 0.52	18.9 ± 2.0	3.2 ± 3.8	1.3 ± 1.3
Pearson correlation coefficient (p-value) with ARG concen- trations	-0.92 (p<0.05)	0.69 (p>0.05)	0.65 (p>0.05)	0.60 (p>0.05)	0.60 (p>0.05)	0.62 (p>0.05)	0.57 (p>0.05)	0.95 (p<0.05)

Table 2. BNR plant water quality data and Pearson correlation coefficients (average results $\pm$ standard deviations from two sampling events).

	Ammonia (mg/L)	TN (mg/L)	TSS (mg/L)	VSS (mg/L)	COD (mg/L)	TP (mg/L)	CBOD ₅ (mg/L)
Raw influent	26.2 ± 6.22	39.4 ± 13.3	309.5 ± 16.3	215.15 ± 5.44	607.0 ± 504.87	31.6 ± 25.7	302.3 ± 215.00
Primary Clarifier	14.7 ± 2.40	24.7 ± 2.92	70.8 ± 35.6	55.5 ± 29.00	327.3 ± 228.16	15.4 ± 2.07	225.8 ± 2.37
BNR	0.66 ± 0.39	250.5 ± 126.6	3201.5 ± 1839.2	1933.8 ± 690.5	1369.5 ± 976.5	>186.0	462.3 ± 81.3
Secondary Clarifier	0.11 ± 0.10	3.6 ± 0.57	3.3 ± 1.27	4.3 ± 2.97	40.0 ± 11.3	0.10 ± 0.07	6.5 ± 4.16
UV	0.14 ± 0.15	4.0 ± 0.57	3.4 ± 1.70	1.6 ± 0.85	18.0 ± 2.83	0.00 ± 0.00	5.6 ± 3.45
Pearson correlation coefficient (p-value) with ARG concentrations	0.83 (p>0.05)	0.002 (p>0.05)	0.005 (p>0.05)	-0.0001 (p>0.05)	0.32 (p>0.05)	0.0035 (p>0.05)	0.34 (p>0.05)

Figure 1
[Click here to download Figure: Figure 1.docx](#)

Figure 1. Schematic layout of the treatment trains of A) the aerated lagoon (AL) plant and B) the biological nutrient removal (BNR) plant with sampling sites indicated by letters (A-E).

Figure 2
[Click here to download high resolution image](#)

Figure 3
[Click here to download high resolution image](#)